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INTERFERENCE BETWEEN *COXIELLA BURNETI* AND CYTOPATHIC VIRUSES IN CHICK EMBRYO CELL CULTURES. I. ESTABLISHMENT OF THE INTERFERENCE

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Summary. — The propagation of *Coxiella burneti* in chick embryo cell (CEC) cultures interfered with the growth of some cytopathic viruses with which the cultures were subsequently challenged. This interference was the most pronounced with Sindbis, Western equine encephalomyelitis (WEE) and vesicular stomatitis (VS) viruses, and less so with pseudorabies and vaccinia viruses. No interference was observed between *C. burneti* and Newcastle disease virus (NDV). The interference was manifested by prevention of viral cytopathic effect (CPE), reduction of virus yield and reduction of plaque number and size. The onset and the degree of interference depended on the extent of *C. burneti* multiplication, which was determined by the multiplicity of infection with *C. burneti* and the time elapsed from inoculation of rickettsiae till challenge. Although the degree of interference decreased with increasing dose of challenging virus, some interference was still observed with a high dose of challenging virus.

Introduction

While attention has been paid for a long time to the interference between rickettsiae (Price, 1953; Price *et al.*, 1954) and between bacteria and rickettsiae (Mika *et al.*, 1955, 1958, 1959; Victor *et al.*, 1955; Pirsch and Mika, 1959) or vice versa (Owen and Larson, 1956), studies on the interactions between rickettsiae and viruses in the process of the mixed infection have been started only recently. There are reports on the interference between rickettsiae and viruses in vitro (Tribble *et al.*, 1965) and between viruses and rickettsiae in vivo (Janssen *et al.*, 1965); moreover, the ability of rickettsiae to induce interferon in vitro (Hopps *et al.*, 1964; Hahon and Kozikowski, 1968) and in vivo (Kazár, 1966) was also reported.

The present work was concerned with the interference between *C. burneti* and some cytopathic viruses in CEC cultures, mainly with the temperature, time and quantitative aspects of this interference.

Materials and Methods

Cell cultures and media. Primary cultures of CEC were prepared from 11 day-old chick embryos according to the method of Dulbecco (1952) with minor modifications. Cultures were seeded with suspensions containing 0.5—1.0 × 10^6 cells/ml and grown in medium 199 supplemented

with 10% of heated calf serum. The maintenance medium contained only 2% of serum. Antibiotics, namely 200 units/ml of penicillin and 200 μg/ml of streptomycin, were added to the media only in virus titrations and preparation of stock virus.

C. burneti. Stock materials were prepared as 10% yolk sac suspensions in medium 199 from chick embryos infected with strains Nine Mile and Henzerling (serologically in phase II) or Florián, L-35 and Vančo (serologically in phase I) and stored at —60° C.

Viruses. Sindbis virus; strain No. 15 of WEE virus; the BUK strain of pseudorabies virus; and the Hertfordshire strain of NDV (the latter cloned by Dr. J. Závada from the Institute of Virology in Bratislava) were grown in CEC cultures; the Indiana strain of VS virus was propagated in L cells; all these viruses originated from the collection of the Institute of Virology, Bratislava. The WR strain of vaccinia virus obtained originally from the laboratory of Dr. Salzman (National Institute of Allergy and Infectious Diseases, Bethesda, Maryland) was maintained in HeLa cells. All viruses were stored at —60° C.

C. burneti titration. Six to seven days old chick embryos were infected with 0.25 ml of tenfold dilutions of stock material of the respective strain of *C. burneti*. Preparations of yolk sacs harvested 9—10 days after inoculation of *C. burneti* were examined microscopically after staining by the method of Gimenez (1964) for the presence of *C. burneti*. The EID_{50} values were estimated from quadruplicates of all dilutions according to the formula of Reed and Muench.

Estimation of the extent of multiplication of C. burneti in CEC cultures. 48 hours old CEC cultures grown on coverslips in tubes were freed of medium and infected with 1 ml of the given dilution of rickettsial stock material. At the same time the input multiplicity of infection was estimated. After 2—2.5 hours of adsorption at 37° C, the inoculum was removed, the cultures washed thrice with phosphate buffered saline (PBS), supplied with maintenance medium and incubated further at 32° or 37° C. At certain time intervals after the end of the adsorption of *C. burneti* the coverslip cultures were withdrawn, dried on air, stained according to Giemsa-Romanowsky and the extent of multiplication of *C. burneti* was determined by calculating the proportion of cells containing in demarcated vacuoles clearly visible rickettsiae. Two hundred cells were evaluated from each culture.

Virus titration. Tenfold dilutions of Sindbis, WEE, VS, NDV and pseudorabies viruses were inoculated in 0.1 ml volumes into CEC tube cultures (quadruplicates and duplicates were used for estimation of input virus doses and virus yields, respectively). The CPE was read after 48 hours and the virus titres were expressed in $TCID_{50}$ values calculated according to Reed and Muench. Vaccinia virus was titrated by the plaque method by inoculating 0.3 ml of virus dilutions into 4 cm Petri dish CEC cultures in triplicates. After 1 hour of virus adsorption at 37° C, the cultures were overlaid with 1% agar in medium 199 supplemented with 6% heated calf serum. Three days later the cultures were fixed with 10% formalin for 2 hours, the agar overlay was removed, and the cultures were washed with water and stained with 1% methylene blue for 1 hour; the plaques were counted and the virus titres expressed in PFU values.

Estimation of the extent of interference between C. burneti and viruses. Twenty-four or 48 hours after inoculation with *C. burneti* (at the time of estimation of *C. burneti* multiplication), CEC cultures grown in tubes without coverslips were freed of medium, washed with PBS and infected with 200 $TCID_{50}$ or 200 PFU of virus in 2 ml of fresh maintenance medium. Forty-eight hours later the medium was withdrawn from triplicate tubes, pooled and stored at —30° C until titrated. The virus yields were estimated by titration in fresh CEC cultures. In parallel experiments, using cultures of similar age, but not infected with *C. burneti*, the virus yields were estimated at the same intervals. The extent of interference between *C. burneti* and the given cytopathic virus was expressed as the interference index representing the ratio of the virus yield from *C. burneti*-infected cultures to that from control cultures. For sake of simplicity, the interference indices were expressed in logarithmic values.

Results

The inhibition of the CPE of Sindbis virus in CEC cultures infected with C. burneti *and the reduction of Sindbis virus yield from these cells*

CEC tube cultures were infected with the Nine Mile strain of *C. burneti* at the multiplicity infection (MI) of 20 and 2 EID_{50} per cell, respectivety. After 2, 4 and 6 days of incubation at 32° C, the cultures were superinfected

with 1000 $TCID_{50}$ of Sindbis virus and cultivated further at 37° C. The same dose of virus was inoculated at the same time intervals into control CEC cultures, namely cells not infected with *C. burneti* and cells treated with a 10^{-1} dilution of 10% yolk sac suspension prepared from uninfected chick

Table 1. Inhibition of Sindbis virus CPE in CEC cultures infected with C. burneti and reduction of Sindbis virus yield from these cultures

CEC cultures treated on day 0 with	CPE* and titres (log $TCID_{50}$/ml)** of Sindbis virus in cultures infected with 1000 $TCID_{50}$ of Sindbis virus on		
	day 2	day 4	day 6
C. burneti, 20 EID_{50}/cell	+++ 8.5	+ 6.0	+ 4.0
C. burneti, 0.2 EID/cell	+++ 9.0	+++ 8.5	++ 7.5
10% yolk sac suspension from uninfected chick embryos	+++ 9.0	+++ 9.0	+++ 9.0
Untreated controls	+++ 9.0	+++ 9.0	+++ 8.5

* +, ++, +++ refer to partial, incomplete and complete CPE, respectively.
** The titres of Sindbis virus were determin ed 48 hours after challenge, i.e. on days, 4, 6 and 8, respectively.

embryos. The CPE was read 48 hours after virus inoculation usualy in triplicate tubes, from which 0.5 ml portions of medium were simultaneously withdrawn for assay of virus yield.

As distinct from control CEC cultures not infected with *C. burneti* which showed a complete CPE, the cultures infected for 6 days with a low dose of *C. burneti* displayed an incomplete CPE and those infected with a high dose of *C. burneti* showed only a partial CPE already from the 4th day of rickettsial infection (Table 1). The partial inhibition of the CPE was accompanied by a reduction of Sindbis virus yield from cells pre-infected with *C. burneti*. The treatment of CEC cultures with a suspension prepared from

Table 2. Influence of temperature of cultivation of C. burneti-infected CEC on the extent of interference between C. burneti and Sindbis virus

Cultivation temperature °C		Days after inoculation with *C. burneti*				
		1	2	3	4	5
32	%	6	19	47	68	86
	I	0	0	−1.5	−2.5	−4
37	%	9	26	58	75	89
	I	0	0	−2	−3	−4

% = per cent of *C. burneti*-infected CEC at the time of their superinfection with 1000 $TCID_{50}$ of Sindbis virus.
I = interference index in log values.

non-infective yolk sacs or infection of CEC even with a high dose of *C. burneti* for only 2 days exerted no influence either on the degree of the CPE or on the yield of Sindbis virus.

The influence of the temperature of cultivation of C. burneti *— infected CEC cultures on the extent of interference between* C. burneti *and Sindbis virus*

A dose of 1000 $TCID_{50}$ of Sindbis virus was added to CEC cultures pre-infected with the Nine Mile strain of *C. burneti* for 1, 2, 3, 4 and 5 days at the MI of 20 EID_{50} per cell at 32° or 37° C. The same virus dose was inoculated into control CEC cultures not infected with *C. burneti*. The incubation after virus infection proceeded at 37° C. Samples of media were withdrawn 48 hours later for assay of virus yield and interference index. At the time of virus inoculation, the degree of multiplication of *C. burneti* was determined in coverslip cultures from parallel tubes.

The temperature of incubation of CEC cultures during their infection with *C. burneti* did not exert any substantial influence on the extent of interference (Table 2). A slightly more pronounced interference was observed in cultures maintained at 37° C. Since a longer incubation of cultures without a change of medium at this temperature led in some cases to a partial degeneration of cells, the temperature of 32° C was chosen for the induction of interference with *C. burneti* in further experiments.

Table 3. Dependence of the extent of interference between C. burneti and WEE virus in CEC cultures on the degree of multiplication of C. burneti

Multiplicity of infection with *C. burneti*	Days after inoculation with *C. burneti*					
	2		4		6	
	%	I	%	I	%	I
60	34	−1.5	88	−6	96	−8
20	23	0	81	−5	92	−7.5
6	17	0	72	−4	84	−6
2	9	0	48	−2.5	63	−4
0.6	3	0	31	−1	51	−2.5
0.2	1	0	13	0	33	−1.5
0.06	0	0	4	0	11	0

% = per cent of *C. burneti*-infected CEC at the time of their superinfection with 200 $TCID_{50}$ of WEE virus.
I = interference index in log values.

The dependence of the extent of interference between C. burneti *and WEE virus in CEC cultures on the degree of multiplication of* C. burneti

The preceding experiments revealed a certain dependence of the extent of interference between *C. burneti* and Sindbis virus in CEC cultures on the degree of multiplication of *C. burneti* in these cultures at the time of virus inoculation. Assuming that the degree of multiplication of *C. burneti* at a certain time interval after virus infection of CEC cultures depends not

only on the duration of the rickettsial infection but also on the infecting rickettsial dose, we investigated the effect of the dose of *C. burneti* and the duration of the rickettsial infection on the propagation of *C. burneti* and its ability to interfere in CEC cultures with WEE virus, another representative of the arbovirus group.

Tube cultures were infected with 1 ml doses of *C. burneti* at the multiplicity range from 0.06—60 EID_{50} per cell. After 2, 4 and 6 days both the infected and the control uninfected cultures were challenged with 200 CPD_{50} of WEE virus. At the same time intervals, the proportion of cells infected with *C. burneti* in the culture was estimated. Forty-eight hours after virus inoculation, the virus yield and the interference index were assayed.

Table 3 clearly demonstrates the dependence of the extent of interference between *C. burneti* and WEE virus on the degree of multiplication of *C. burneti*; the latter depends on the multiplicity of infection with *C. burneti* and the time which elapsed between the rickettsial and viral inoculations.

In the CEC cultures infected with a large dose of *C. burneti* (MI of 60 EID_{50} per cell), the interference with WEE virus could already be observed when the cultures were challenged 2 days after infection with rickettsiae. If added only 6 days after infection with rickettsiae, WEE virus did not multiply at all. However, with a large dose of *C. burneti*, the CEC cultures showed 6 days after infection a partial degeneration due to the rickettsial infection itself. With moderate doses of *C. burneti* (MI = 2—20 EID_{50}/cell), a marked interference was found with WEE virus inoculated 4 days after infection with *C. burneti*. In cultures infected with small doses of *C. burneti* (MI below 1 EID_{50}/cell), the interference was manifested when the cultures were challenged with WEE virus 4—6 days after inoculation of *C. burneti*, when at least 1/3 of the cells in the culture were actually infected with *C. burneti*.

Table 4. Ability of various C. burneti strains to induce the interference with WEE virus in CEC cultures

C. burneti strain	MI	%	I
Florián	2	38	−1.5
L-35	2	44	−2
Vančo	6	59	−3
Nine Mile	6	62	−3
Henzerling	10	71	−3.5

MI = input multiplicity of infection with *C. burneti*.
% = per cent of CEC infected with *C. burneti* 4 days after inoculation, when the cultures were superinfected with 200 $TCID_{50}$ of WEE virus.
I = interference index in log values.

The ability of various strains of C. burneti *to induce in CEC cultures the interference with WEE virus*

To compare the ability of various strains of *C. burneti* to induce in CEC cultures the interference with cytopathic viruses, we infected the cultures

with moderate doses (MI = 2—10 EID_{50}/cell) of strains Nine Mile, Henzerling, Vančo, Florián and L-35 of *C. burneti*. Four days after infection with *C. burneti*, the cultures were challenged with 200 $TCID_{50}$ of WEE virus. Forty-eight hours later samples of media were withdrawn from control and *C. burneti*-infected CEC cultures for the assay of WEE virus yields.

As shown in Table 4, there was no marked difference among the various strains of *C. burneti* in their ability to induce in CEC cultures the interference with WEE virus. Slight differences in the extent of interference induced with various strains of *C. burneti* were probably due to a different degree of multiplication of these strains in CEC cultures at the time of virus inoculation.

Table 5. Effect of the dose of superinfecting virus on the extent of interference between C. burneti and various viruses in CEC cultures

Virus and its dose (log $TCID_{50}$)		Interference index with *C. burneti* strains	
		Henzerling (MI = 20)	Nine Mile (MI = 6)
Sindbis	2.3	−4.5	−3
	4.3	−2.5	−2
	6.3	−1.5	−1
WEE	2.0	−5	−3.5
	4.0	−3	−2
	6.0	−2	−1.5
VS	2.3	−4	−3
	4.3	−2	−2
	6.3	−2	−1.5

The CEC cultures were superinfected with the indicated viruses 4 days after inoculation with *C. burneti*.

The influence of the dose of challenging virus on the extent of interference between C. burneti *and viruses in CEC cultures*

Four days after infection with the strain Nine Mile (MI = 6 EID_{50}/cell) or Henzerling (MI = 20 EID_{50}/cell), CEC cultures were superinfected with increasing doses of Sindbis, WEE or VS viruses, which were inoculated at the same time into parallel control CEC cultures of the same age. The extent of the interference was evaluated as before.

Although the increasing dose of superinfecting virus led to a decrease of the extent of interference, the latter could be demonstrated even with large doses of superinfecting virus (Table 5).

The comparison of the ability of C. burneti *to interfere with various cytopathic viruses*

In the attempt to test the influence of preinfection of CEC cultures with *C. burneti* on the multiplication of various viruses in these cultures, we challenged cultures infected with *C. burneti* for 4 days (MI = 60, 20 and

6 EID_{50}/cell) with 200 $TCID_{50}$ of Sindbis, WEE, VS, pseudorabies and NDV viruses or 200 PFU of vaccinia virus, respectively. Control CEC cultures of the same age were infected with similar virus doses and the extent of interference was estimated as before.

The results (Table 6) confirmed a different ability of the viruses tested to grow in CEC cultures preinfected with *C. burneti*. While there was an expressed inhibition of multiplication of Sindbis, WEE and VS viruses, the

Table 6. Comparison of the interfering ability of C. burneti with various viruses

Virus	Interference index in CEC cultures infected with *C. burneti* at		
	MI = 60	MI = 20	MI = 6
Sindbis	−6	−4	−3
WEE	−6	−5	−4
VS	−5.5	−4	−3
NDV	−0.5	0	0
Pseudorabies	−2	−1.5	−1
Vaccinia	−1	−1	−0.5

The CEC cultures were superinfected with 200 CPD_{50} of the indicated viruses (200 PFU in the case of vaccinia virus) 4 days after inoculation with *C. burneti*.

propagation of pseudorabies and vaccinia viruses was blocked much less and NDV grew equally well in *C. burneti* — infected and control CEC cultures.

Besides reducing the virus yield in CEC cultures preinfected with *C. burneti*, the interference was also manifested by an inhibition of the CPE caused by 200 $TCID_{50}$ of the respective virus in these cultures (Figs 1—4).

The inhibition of plaque formation by viruses in CEC cultures preinfected with C. burneti

In the preceding experiments the interference between *C. burneti* and viruses in CEC cultures was quantitatively demonstrated by differences in virus yields from control and *C. burneti* — infected cultures. In the following experiments we investigated the influence of *C. burneti* infection of CEC cultures on the ability of these cultures to form plaques after virus infection.

CEC cultures grown in 6 cm Petri dishes were infected with 1 ml of tenfold dilutions of the Nine Mile strain of *C. burneti* (MI = 20, 2 and 0.2 EID_{50}/cell). After 2—2.5 hours of adsorption at 37° C, the cultures were thrice washed and further incubated in a hermetically closed glass container at 32° C. In cultures infected with *C. burneti* for 4 day and control cultures of the same age, we titrated Sindbis, WEE, VS, pseudorabies, vaccenia and NDV viruses by inoculating 0.5 ml of tenfold virus dilutions into triplicate plates.

Table 7 shows that the plaque formation by viruses in CEC cultures infected with *C. burneti* was inhibited. The effect was mostly pronounced for Sindbis, WEE and VS viruses, and less for pseudorabies and vaccinia viruses. The plaque formation with NDV was only slightly affected by *C. burneti*

Table 7. Inhibition of the plaque formation with various viruses in CEC cultures infected with C. burneti

Virus	Virus titres (PFU/ml) in			
	control CEC	CEC infected with *C. burneti* at		
		MI = 20	MI = 2	MI = 0.2
Sindbis	3.2×10^8	?	1.8×10^7	2.8×10^8
WEE	4.7×10^7	?	2.4×10^6	3.1×10^7
VS	2.1×10^7	—	6.4×10^6	—
Pseudorabies	1.6×10^8	2.1×10^7	—	—
Vaccinia	6.2×10^6	3.6×10^5	—	—
NDV	1.7×10^7	9.1×10^6	1.3×10^7	—

? The exact plaque count could not be determined.
— Not done.

infection of CEC cultures. The inhibition of plaque formation was again dependent on the infecting dose of *C. burneti*. In cultures infected with a small dose of *C. burneti* (MI = 0.2 EID_{50}/cell) the plaque counts of Sindbis and WEE viruses were lowered only slightly, but in cultures infected with a moderate dose of *C. burneti* the reduction was more pronounced.

The preinfection of CEC cultures with *C. burneti* caused not only the reduction of the plaque count, but it also influenced the plaque size: it reduced the diameter of Sindbis, WEE, VS and pseudorabies virus plaques 3—10 times as compared to that observed in control cultures not infected with *C. burneti*. In cultures preinfected with a large dose of *C. burneti* (MI = 20 EID_{50}/cell), the plaques of Sindbis and WEE viruses were so small as to be discernible only under the microscope, so that it was difficult to estimate exactly the plaque counts.

Discussion

The questions of the mutual relationships between rickettsiae and viruses in the mixed infection began to attract the attention only recently and the knowledge obtained hitherto is fragmentary and incongruous. Although there were reports on the interference between rickettsiae and viruses (Tribble *et al.*, 1965) or viruses and rickettsiae (Janssen *et al.*, 1965), this phenomenon was not analyzed in detail and the mechanism of interference remained obscure. The interference between rickettsiae and viruses, however, may be of practical significance, as revealed the observation of Burgdorfer (cited by Philip, 1961) that an interference between Colorado fever virus and *R. rickettsii* occurs under certain contitions of the mixed infection of their natural vector, the tick *Dermacentor andersoni*.

The most suitable substrate for the study of quantitative and time factors in the mixed infection with two viruses are tissue cultures. This model appeared to be useful also for the investigation of the mixed rickettsial and viral infection especially in the case of *C. burneti*. The ability of *C. burneti* to multiply in demarcated vacuoles of cells has been exploited for the assay

of the degree of multiplication of *C. burneti* in various tissue cultures (Weiss and Pietryk, 1956; Roberts and Downs, 1959). CEC cultures are especially suitable for this purpose, because they support rather well the multiplication of *C. burneti* without any marked cytopathic changes and many viruses cause in these cultures a distinct CPE and plaque formation, so that the possible interference can be quantitatively evaluated.

The use of varying infecting doses and different time intervals between the inoculation of *C. burneti* and a virus made it possible to reveal a significant dependence of the extent of interference between *C. burneti* and the given virus on the degree of multiplication of *C. burneti* in cells at the time of virus challenge.

The small differences in the extent of interference with various strains of *C. burneti* were due to the various degree of multiplication of these *C. burneti* strains in CEC cultures rather than to the intrinsic properties of these strains. Of certain importance is the observation that also strains of *C. burneti* in phase I, in which they exist in nature (Stoker and Fiset, 1956), are able to induce the interference, provided they are allowed to multiply sufficiently in CEC cultures.

Since Pinkerton and Hass (1932) found the beneficial effect of a lower cultivation temperature on the propagation of rickettsiae in vitro, the temperatures of 32—35° C have been frequently used in experiments on the cultivation of rickettsiae in tissue cultures. However, we did not observe in our experiments a reduced propagation of *C. burneti* at 37° C as compared with 32° C. On the contraty, *C. burneti* multiplied in CEC cultures at 37° C somewhat faster and more intensively than at 32° C, what corresponded also to a slightly increased extent of interference at 37° C.

Although there appeared a certain dependence of the extent of interference on the dose of superinfecting virus, it was impossible to overcome the interference between *C. burneti* and viruses by the use of even extremely large virus doses. It seems likely that the interference has the "all or none" character and that the establishment or absence of the interference are determined more by the degree of multiplication of *C. burneti* in the cell than by the dose of superinfecting virus.

The interference between *C. burneti* and some cytopathic viruses was manifested not only by the inhibition of the CPE and the reduction of virus yields from CEC cultures infected with *C. burneti*, but also by the reduction of plaque counts and size directly in CEC cultures preinfected with *C. burneti*. This implies that certain proportion of cells in the culture is unable to support the multiplication of some virus s due to the rickettsial infection. The differences in the ability of the tested viruses (Sindbis, WEE, VS, pseudorabies, vaccinia and NDV) to grow in CEC cultures preinfected with *C. burneti* are probably caused by different metabolic requirements of these viruses and different localization of their synthesis in cells. E. g., NDV which multiplies equally well in *C. burneti* — infected and uninfected control CEC cultures, is able to grow quite well in CEC cultures UV-irradiated with a dose 10 times higher than that sufficient to stop the cell multiplication and to cause 75—90% inhibition of cellular RNA synthesis (Rosenberg

and Rosenbergová, 1962). An important role may be played also by the different sensitivity of the viruses tested to the inhibitory activity of interferon. Those viruses (Sindbis, WEE and VS) which were most markedly inhibited in their multiplication are also the most sensitive to the effect of interferon. On the other hand, the inhibition of multiplication of NDV which is poorly sensitive to interferon (Baron, 1964) was only negligible in *C. burneti*-infected CEC cultures.

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Explanation of Photomicrographs:

Fig. 1. CPE in control CEC cultures 48 hours after infection with 200 $TCID_{50}$ of WEE virus
Fig. 2. Inhibition of CPE in CEC cultures first infected for 4 days with *C. burneti* and then superinfected with 200 $TCID_{50}$ of WEE virus for 48 hours.
Fig. 3. CPE in control CEC cultures 48 hours after infection with 200 $TCID_{50}$ of VS virus.
Fig. 4. Inhibition of CPE in CEC cultures first infected for 4 days with *C. burneti* and then superinfected with 200 $TCID_{50}$ of VS virus for 48 hours.